

**MECHANISMS OF INDIVIDUAL VARIATION IN REPRODUCTIVE BEHAVIOR IN
FEMALE *Xiphophorous birchmanni***

A Thesis

by

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ABSTRACT

Mate choice is a powerful driver of sexual selection, and can influence both the strength and direction of selection on phenotypic traits within a population. In *Xiphophorus birchmanni*, female mate preferences have been extensively characterized. The majority of studies in this species have focused on documenting a mean value for female mate preference within a population. Despite the rich history of mate choice research in this genus and the apparent importance of studying mechanisms of female mate choice for understanding sexual selection processes, no studies to-date have investigated the hormonal mechanisms that underlie female mate choice behavior in this species. Because of its role in reproductive processes across taxa, cortisol was selected as a candidate hormone for involvement in the modulation of reproductive behavior. To establish baseline cortisol values and evaluate possible relationships between cortisol and reproductive activity in this viviparous species, I first evaluated consistency and repeatability of reproductive behavior and water-borne cortisol levels in captive *X. birchmanni* over six days of repeated mate choice testing. Repeatability and consistency of mate preferences were low, with low volume water sampling for cortisol appearing to be a potential stressor. To determine if repeated sampling could reduce sampling stress, I collected water from females placed in larger volume collection beakers over 4 consecutive days. Water-borne cortisol levels decreased over the sampling period, suggesting habituation to the repeated sampling procedure. Although cortisol values fell within the range of other poeciliid fishes, data indicated that plasma and water-borne cortisol levels were not correlated in this species. Finally, to determine if a relationship existed between circulating cortisol levels and reproductive status in a wild population of *X. birchmanni* I collected plasma and ovarian samples from wild caught females and correlated reproductive status with plasma cortisol levels. I found that cortisol levels vary with reproductive status. Although sample sizes were small, cortisol was elevated during gestation, a pattern similar to guppies and progesterone in mammals. Results suggested that cortisol may play a role in pregnancy and reproductive behavior in the species and that if water-borne hormone sampling could be validated, this species might serve as a useful model system for teleost behavioral endocrinology.

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CHAPTER I

INTRODUCTION

Mate choice is a powerful driver of sexual selection, and can influence both the strength and direction of selection on phenotypic traits within a population[1-3]. Traditionally, the study of mate choice is concerned with documenting population-level female preferences for male traits based on female mate choice behavior [4]. Any variation in female preference surrounding the population mean has generally been considered 'noise' and dismissed from further analysis [1,5]. Within species and even within individuals, mate choice behavior can be highly variable and can change across an individual's lifetime [1,5,6]. Individual differences in mate choice behaviors may influence the strength of sexual selection in multiple ways. For example, females that display a low degree of receptivity will either be more likely or less likely to mate randomly than those females with a higher degree of receptive behaviors. If these females are more likely to mate at random then low levels of receptive behavior will be associated with weak sexual selection on male traits; if, however, females with low levels of receptive behavior are instead *more* choosy than females with high levels of receptive behavior, then low levels of receptive behavior will be associated with strong sexual selection on male traits. Changes in reproductive behavior may occur according to ecological factors like predation and environmental disturbance or according to physiological condition [7-10]. Thus, understanding proximate mechanisms underlying female mate choice behaviors is important for understanding sexual selection and the evolution of mate choice behaviors.

Swordtails (Teleostei: *Xiphophorus*) are a long-studied model in the evolution and genetics of sexual communication[11,12]. *Xiphophorus birchmanni* are ovoviviparous internal fertilizers, have a year-round breeding season, and the females can store sperm for up to ten months. This species displays sexually dimorphic phenotypes, with small females having little ornamentation and larger males having prominent dorsal fins, vertical barring on the body and fleshy nuchal humps. These fish are readily collectable, amenable to experimental manipulation and display robust and easily quantifiable behaviors.

In *X. birchmanni*, female mate preferences have been extensively characterized [13-18], but despite the rich history of mate choice research in this genus and the apparent importance of studying mechanisms of female mate choice for understanding sexual selection processes, the majority of sexual selection studies in this species to date have focused on documenting a mean value for female preference functions within a population. As a result, very little is known about proximate mechanisms underlying individual differences in female mate choice behaviors in *Xiphophorus*.

Across vertebrate taxa, steroid hormones are implicated in coordinating external environmental cues with internal physiological status to inform behavioral output [19-21]. A key initiator of behavioral responses to many social and environmental changes is the rapid increase in circulating plasma levels of glucocorticoid hormones. The major glucocorticoid hormone in teleost fish is cortisol [22-24]. Reproductive behavior and physiology can be suppressed by high levels of cortisol [25] while low levels of circulating cortisol may facilitate reproductive behavior and physiological function [26,27]. In addition, levels of excreted cortisol and expression of genes associated with the stress axis have been shown to vary between fish that display consistency in behavior over time and/or across contexts. This behavioral consistency has been defined as personality [7,28]. These individual differences in personality, correlated with differences in the stress axis suggest that the stress axis could have a long-term role in behavior, which may contribute to individual differences in mate choice behaviors [29]. To my knowledge, no studies to-date have investigated the effect of female circulating cortisol on female mate choice behavior. Individual differences in cortisol levels may be associated with individual differences in reproductive physiology and behavior among females and these differences may lead to changes in the strength and direction of sexual selection on male traits.

In order to investigate the possible role of cortisol in modulating reproductive behavior in female *X. birchmanni* we must first have an understanding of the range of physiological response of cortisol for this species. Understanding physiological ranges for cortisol is not only important for understanding what is high and what is low in terms of cortisol release in a given species but also because cortisol responsiveness may be more important than either basal or peak values of cortisol precisely because of individual

variation in these values. We must also attempt to understand the relationship between cortisol and reproductive behaviors as well as the relationship between cortisol levels and reproductive status. To this end, my study has three objectives: To establish the physiological range of water-borne cortisol in female swordtails, to evaluate possible relationships between cortisol and consistency/repeatability of reproductive behavior in captive animals, and to examine the relationship between cortisol levels, and reproductive status in a natural population.

CHAPTER II

REPEATABILITY AND CONSISTENCY OF MATE CHOICE BEHAVIORS IN FEMALE

X. birchmanni

Historically, research regarding female mate choice has focused on documenting a population preference mean or mean preference function for a given male phenotype that is considered to be the phenotypic optimum (by nature of the fact that this trait is connected either directly or indirectly to some beneficial quality of the mate which leads to increased fitness for the choosy sex)[1]. Any variation in preference surrounding the optimal mean has generally been considered 'noise' and dismissed from further analysis [1,5]. This 'noise' constitutes individual differences among females in their preference behavior. In many studies females who spend more than a pre-determined amount of time in a given area of the choice trial chamber are defined as unresponsive and excluded from analysis [17]. Individual variations in reproductive behavior, when consistent across time and/or context, are defined in the animal behavior literature as animal personalities or behavioral syndromes [7,[19,21]. Researchers in the area of evolution and behavioral ecology have begun to recognize that individual differences have the potential to influence the strength and direction of sexual selection on male traits. For example, preferences that stray from the average expected preference value in even a relatively small number of females might lead to the preservation of male phenotypes that differ from the average preferred male phenotype in a population. This is one possible explanation for the maintenance of variation in male traits in highly polymorphic species like *Uta stansburiana* and *Poecilia reticulata* [22,24] and in more extreme cases has the potential to lead to speciation. Studies have demonstrated that these individual differences often display non-random clustering around specific axes, which indicates that the individual differences in behavior themselves have been subject to selection[30]. Recent studies across taxa have revealed that greater than 30% of the phenotypic variance within a given population often stems from consistent individual differences [31].

Consistency in behavior over time or between contexts for a given individual is a major defining feature of 'personality' or 'behavioral syndromes' in the animal personality literature. In order for a behavior to be considered a direct result of an animal's

personality, it must be consistent. In addition, a behavior is rarely considered heritable unless it is highly repeatable (between individuals) [2,3,32]. Consistency can be measured by calculating a coefficient of variation across repeated trials for a given female [6,33]. If certain mate choice behaviors are consistent and repeatable, then it is possible that they are heritable and are contributing to the maintenance of individual differences in female mate choice and male trait polymorphism. Discerning the proximate mechanisms underlying individual differences in female reproductive behavior is important for revealing how and why individual differences in female preference persist and how they contribute to the maintenance of male polymorphism within a population.

One situation in which low repeatability or consistency would not necessarily indicate low heritability is in the case of animals that have cyclic patterns of physiology that contribute to behavioral output. For example, in the female estrous cycle if an animal were in one phase of a cycle during the first measurement of a behavior and in another phase of the cycle in subsequent measurements, then a statistically significant value for consistency would not be expected [34]. Variation in mate choice due to cyclicity could easily be the cause of reported low repeatability or consistency in some species where monitoring female hormonal or reproductive status is not emphasized. This further highlights the necessity of investigating the hormonal mechanisms underlying female reproductive behavior.

Candidate hormones for involvement in modulation of female reproductive behavior are those that are likely to play a role in synchronizing external cues with the female's internal physiological state. This synchronization would allow a female to time behaviors appropriately according to her reproductive status. In fish, the role of cortisol in mate choice and reproductive behavior/physiology has been a topic of considerable research, however, much of this research is focused on the detrimental effects of very high levels of cortisol [27]. Fewer studies have looked at how cortisol levels influence reproductive behavior and of those that have, most focused on how cortisol influences male behavior [35].

While very high and very low cortisol levels have been linked with suppression of

reproductive behavior and/or physiology, more subtle changes in cortisol levels have also been linked with physiological events within the reproductive cycle. In brown trout, an increase in cortisol is associated with ovulation [36]. In one poeciliid species (*P. reticulata*) cortisol levels have been shown to vary over the course of the reproductive cycle, with cortisol elevated just before fertilization and levels at their lowest at fertilization and just prior to parturition [37]. These data indicate a potential role for cortisol in the hormonal mechanism underlying mate choice behavior.

The goal of this experiment is to investigate whether females of *X. birchmanni* show consistency and/or repeatability in their mate choice behaviors and to determine how these behaviors might correlate with cortisol levels. Few studies to-date have investigated the repeatability or consistency of female preference behaviors in fish [20,33,38]. Association time (the amount of time a female spends in close proximity to a given male) is the standard measurement in fish mate choice experiments. Across several species, association time has been established as a strong predictor of whom a female will mate with (Wong et al., 2005; Ryan et al., 1992). In addition, one of the only studies that used swordtails to look at consistency and repeatability of mate choice behavior, Cummings and Mollaghan, (2006) indicated that association time is the most consistent and repeatable estimate of preference for female swordtails [35]. The goal of my experiment was to evaluate possible relationships between cortisol and consistency/repeatability of reproductive behavior in captive *X. birchmanni*. I hypothesized that females with very high or very low values for water-borne cortisol would be consistently less receptive to males, whereas females with intermediate levels of cortisol would be more receptive to males and either more or less consistent in mate choice behavior than females with very high or very low cortisol levels. This would be consistent with my discussion of choosiness behavior in Chapter I.

Methods

Animals

Adult female northern swordtails (*Xiphophorus birchmanni*, N=28) of various ages were obtained from Calnali, Hidalgo Mexico and housed in the laboratory in mixed-sex groups before the experiment began. Two weeks prior the start of the experiment, females were moved into all-female groups of 4 individuals per 10-gallon aquarium maintained at 24°C with adequate shelter (hollow brick pavers) and a photoperiod of 12:12 h light:dark. Fish were allowed 2 weeks to acclimate to the new aquaria and feeding schedule. Fish were fed with either bloodworms or frozen brine shrimp once daily after experimental procedures.

Mate choice trials

In order to examine the repeatability and consistency of mate choice behavior in *Xiphophorus birchmanni*, females were repeatedly tested in daily mate choice trials on 6 consecutive days. Prior to testing females were handled and marked via subcutaneous elastomer injection in the caudal area for individual identification and then placed in water-borne hormone collection beakers for 1 hour to obtain a peak acute stress cortisol measurement. The peak stress measurement provides a reference value for cortisol release during highly stressful events and is useful for comparison to future data from the same animal.

Following elastomer injection and cortisol sample collection females were allowed to recover in their home tanks for 24 hours before mate choice trials began. Females were tested for 6 days with an identical protocol for each day as follows: Individual females were removed from tanks via hand-net and placed into mate choice tanks (75x30x30cm) with computer monitors at either end to display animated male stimuli and a brick in the center of the tank which females could use to escape from male stimuli. All male stimuli were simulated videos of *X. birchmanni* males of identical body size and morphology with the exception of the dorsal fin. One male stimulus displayed an average sized dorsal fin (based on data collected in Wong and Rosenthal 2006) and the other displayed a dorsal fin that was 33% reduced. Male stimuli were consistent between females and across days. The choice tanks were divided into 3 equal zones

(25 cm ea.). Females were placed in the center zone and allowed to acclimate to the tank for 300 seconds with no stimuli displayed (monochromatic screen). 300 seconds of monochromatic screen was followed by 300 seconds simultaneous presentation of male stimuli (average dorsal fin on one side of the tank and 33% reduced dorsal fin on the other). Following the 300 seconds of male stimuli females were exposed to an additional 300 seconds of monochromatic screen and then a subsequent 300 seconds of the same male stimuli displayed on opposite sides from the first presentation to control for any side biases[13,14]. At the conclusion of mate choice trials, all trial videos were watched and association time (time spend in the zone associated with a given male stimulus) was calculated for each male phenotype. Using these association times, I calculated a preference score by subtracting total time spent with the average dorsal fin male from total time spent with the small (33% reduced) dorsal fin male. Thus, a positive score indicates more time spent with the small dorsal fin male and a negative preference score indicates more time spent with the average sized dorsal fin male[17].

Cortisol sampling

In order to investigate possible correlations between free cortisol levels and female mate choice behavior, individual water-born cortisol levels were measured daily after each trial. Directly following each mate choice trial, females were placed in clean, 200ml beakers with 150ml of clean aquarium water for one hour for the purpose of hormone collection. The water-born hormone sampling procedure was first developed in goldfish for measurement of pheromones and had since been modified for use in multiple species [39] [40]. Each beaker was covered with a paper towel and left untouched in a quiet room for the duration of sampling to reduce external stimulation.

Cortisol analysis

All hormones were extracted and eluted using a previously established protocol (Wong et al, 2008). Free steroid hormones were extracted from water by passing through a Sep-Pak C18 solid phase extraction column connected to a vacuum manifold. Ethyl acetate (4ml) was used as the solvent for elution of steroids from the column. This technique should collect only free (and not glucuronidated or sulfated) steroids (Vermeirssen and Scott, 1996). Eluates were dried under nitrogen and re-suspended in

600 microliters EIA buffer. Additional details and validations of the cortisol assay are provided in Chapter 3.

Results

Behavior

Overall I found no significant patterns either within or between females for consistency or repeatability of mate choice. Total time spent with males varied across days [Figure 2.1].

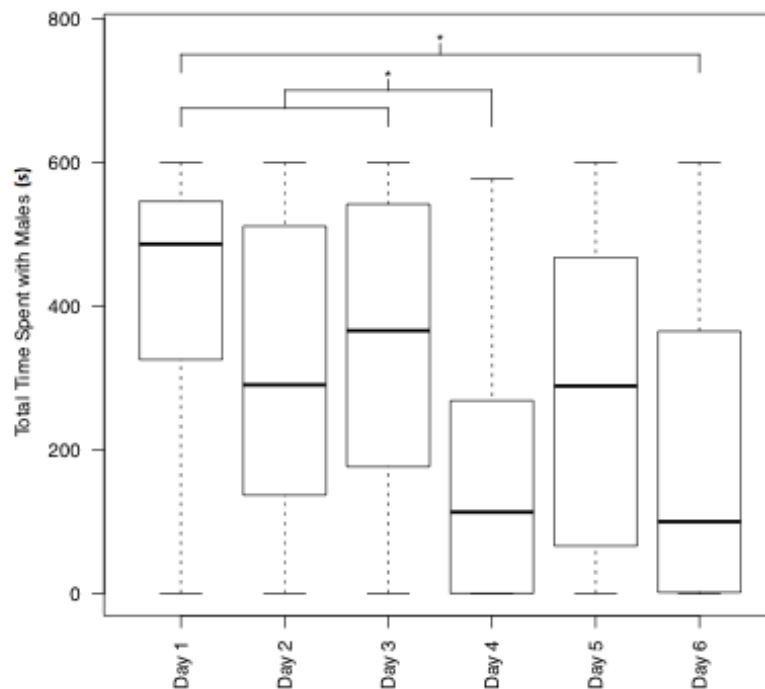


Figure 2.1 Total time spent in male stimulus zones across six days of repeated testing.

When females who chose not to leave the neutral zone were included in the analysis, females showed no significant preference for either male dorsal fin phenotype on most days [Figure 2.2]. Time spent with males on days 4 and 6 showed significant differences from day 1 (0.0017 and 0.0026, respectively), and day 4 additionally

showed a significant difference from day 3 (0.04639) using a Wilcoxon signed rank test and correcting for multiple testing (all days verse all days) using Holm's adjustment.

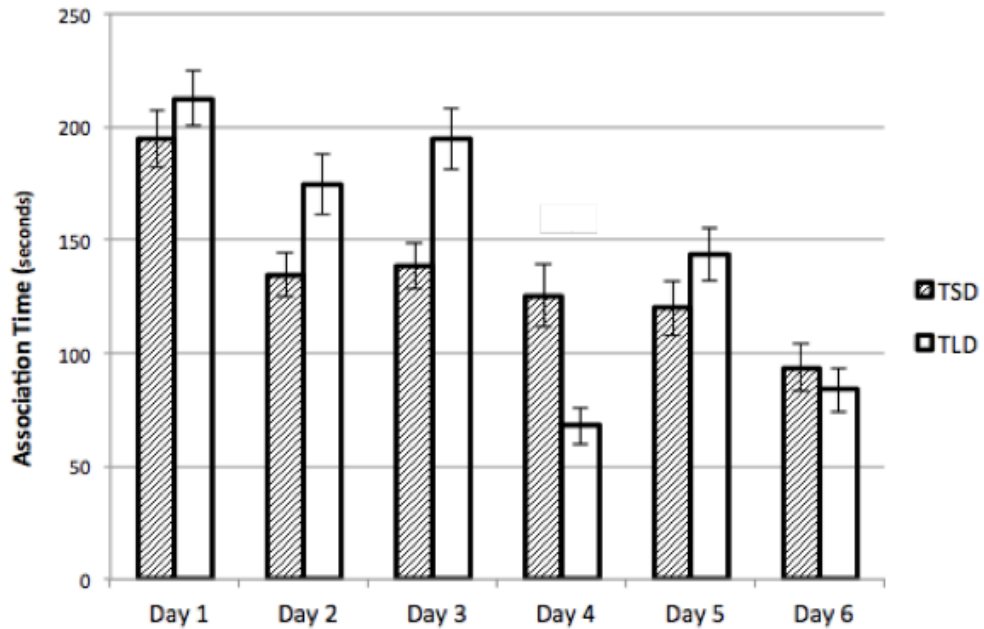


Figure 2.2 Female association times with average (TLD) vs. 33% reduced dorsal fin males (TSD) across 6 days of repeated testing. On day 4 females preferred males with 33% reduced dorsal fins over males with average sized dorsal fins.

When females who did not leave the neutral zone were excluded from the analysis several of the days revealed significant preferences, but individual females did not show significant consistency in preference across repeated testing (repeated measures ANOVA within subjects $F(25, 125)=0.568$, $P=0.9487$) [fig 2.3]

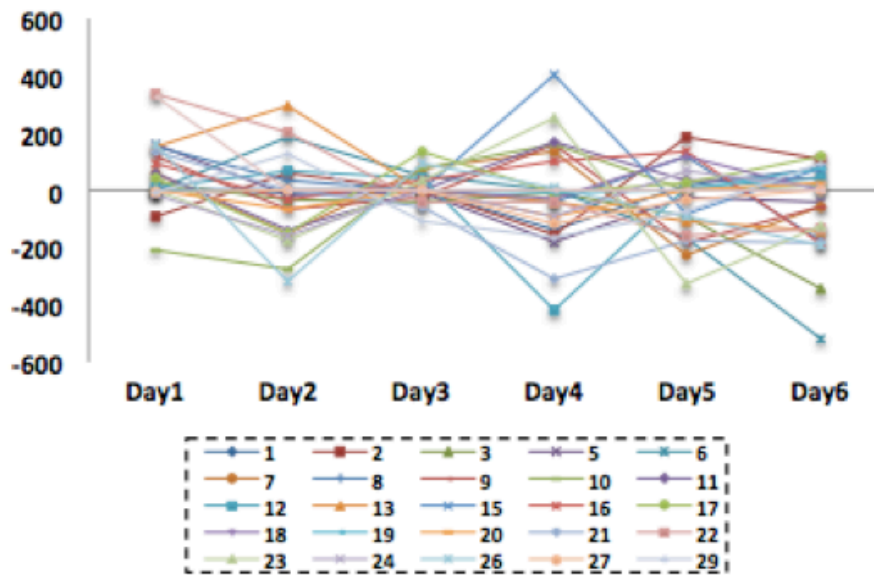


Figure 2.3 Individual preference scores across repeated mate choice trials. Negative scores indicate preference for average sized dorsal finned males, positive scores indicate preference for 33% reduced dorsal finned males.

Cortisol

Few cortisol samples were actually analyzed because of complications with the collection procedure (see Discussion section) but those that were (n=2 fish, 6 total samples analyzed) fell on the standard curve [Fig. 2.4] for the cortisol EIA kit and within the normal range for poeciliid fish [37,41]. In addition, for both females for which the samples were analyzed, there was a significant decrease in waterborne cortisol values from the day of acute stress marking to day 1 of behavioral experiments (Fig. 2.5). This indicates that the behavioral assay itself is probably not a significant stressor.

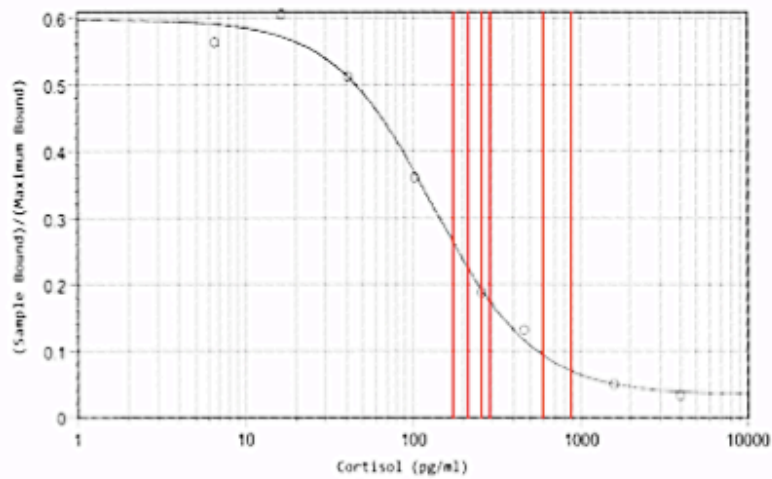


Fig. 2.4 Cortisol samples fall on the standard curve for the Cayman Cortisol EIA kit and within the range of reported values for fish in this genus (red lines represent collected waterborne cortisol sample values).

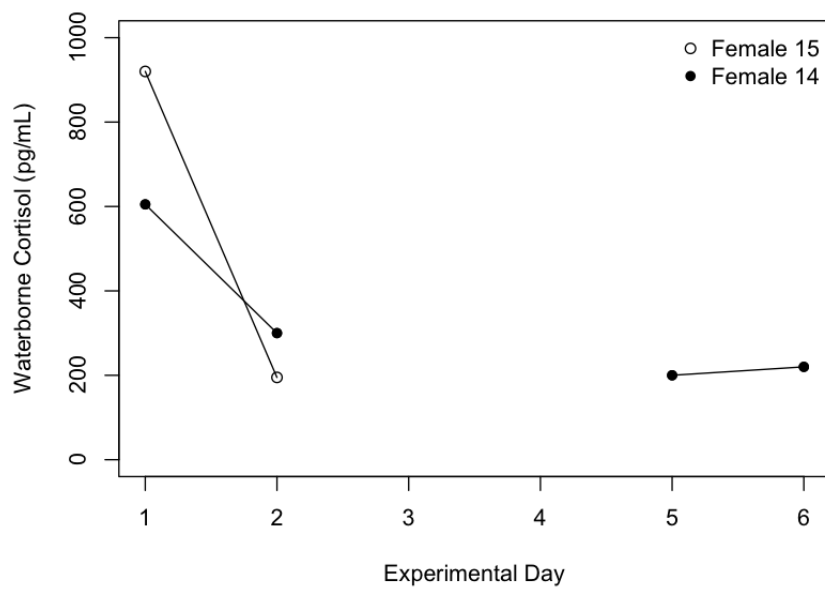


Figure 2.5 Individual female cortisol levels over repeated sampling. Cortisol levels fall within a normal range for those recorded for this genus and show a significant decrease from peak levels on Day 1 to the first day of testing (Day 2).

Discussion

The purpose of this study was to investigate the level of consistency and repeatability for female *X. birchmanni* mate choice preferences and to correlate any patterns in individual mate choice behaviors with cortisol levels. Based on the results of this study, both consistency and repeatability appear to be low for this species.

These results are similar to patterns seen in several previous studies regarding the repeatability of mate choice [3,20,25-27,32,42-44] where repeatability and consistency over repeated trials was low. In one of these studies [44] the researchers gave female red jungle fowl a choice of 2 potential mates. In the first trial, a high proportion of the females initially chose the attractive male (based on experimenter predictions regarding the fitness of a given male) but on subsequent trials a significant proportion of the females chose to mate with the less attractive male. One possible explanation for this is that females mate multiply in order to generate sperm competition and that mate selection is happening at the post copulatory level [23]. This is a potentially valid explanation for variation in preference across repeated trials in *X. birchmanni* as it has been shown that females of this genus can store sperm for up to 8 months within the reproductive tract [45], which would allow for multiple matings and sperm competition to occur over a more extended period.

Interestingly, the only day which females showed a significant preference for a male phenotype was on day 4. On day 4 females, overall, prefer the reduced dorsal fin male to the normal dorsal fin male. This preference for large body size with reduced dorsal fin size is consistent with previous data from this species which indicated that females prefer males with 33% reduced dorsal fins to males with average sized dorsal fins [7,17,28]. It is important to note that the Fisher et al. study was not replicated within a given female so there was no data on the consistency of this preference. Further, an effect of room was also detected on day 4, the only day when females showed a strong significant preference.

Although this study does not have definitive results regarding the repeatability of mate choice and underlying mechanisms in this species, it serves to emphasize several

areas of importance for consideration when approaching future studies of this nature. The broadening of focus within the mate choice literature from attempting to define an optimum male trait value for a given species to including attempts to understand variation in female reproductive behavior within a population is a relatively recent occurrence. Because of this, I think that there are many areas within the existing mate choice paradigms that still need to be modified to better answer questions centering on individual variation in female proceptivity and receptivity.

For example, the most common protocol for pre-choice test trial housing in this genus is a male free environment. Pre-choice trial housing should be a very important consideration during the experimental design process given the fact that several studies suggest that females will actually become *less* choosy when they are isolated from males [46]. Housing females separately from males for extended periods prior to testing might be acceptable if the goal of the experiment is to produce females who associate with males at any cost but I do not believe that this method produces an accurate representation of female choice in natural populations and thus would be of very little utility for understanding the evolution of male traits and female preferences in the wild.

A second component of this experiment that lies within a relatively new realm of study was the water-borne hormone collection technique. This is one of only a few studies that have attempted water-borne hormone collection to date and the first study in *X. birchmanni*. Over the course of this experiment it became evident that the fish were stressed by the hormone collection procedure. Many females, when placed in beakers for hormone collection would repeatedly attempt to jump from the water and ultimately end up on the lab bench. Despite the fact that hormones had been successfully collected for similarly sized fish with similar beaker size and water volumes [29,47], it seemed that this species was not tolerating the experimental parameters well. This is not altogether a surprise, as confinement in small volumes of water is considered a stressor in fish and behavioral ecology plays a role in how different species will react to this type of confinement [11,12,25]. As a result of this observation, I hypothesized that this behavioral response might be a consequence of the volume of water and beaker size being used. Therefore, I chose not analyze all of the cortisol samples associated with this experiment and instead examined increasing beaker size and water volume in

order to determine whether this sampling technique is a viable option in this species. These studies are described in the next chapter.

CHAPTER III

CORTISOL RESPONSE TO REPEATED CONFINEMENT

Introduction

Water-born hormone sampling, a non-invasive technique for measuring steroid hormone concentrations released passively across gills into fish holding water, is becoming a widely used technique in the field of fish behavioral endocrinology. It is of particular utility in studies using small fish in which plasma sampling is often a terminal procedure, in studies in which the goal is to understand long-term patterns of hormone release and also in studies measuring cortisol where the sampling procedure itself may induce a stress response.

Initially, this technique gained the most attention among aquaculture researchers for monitoring and maintaining welfare of commercially important fish[39] and early experiments showed significant correlation between plasma cortisol levels and water-born cortisol levels in stock tanks of aquaculture species like rainbow trout (*Oncorhynchus mykiss*) [41]. More recently, this technique has been adopted by physiologists, endocrinologists and behaviorists [41,47-49] for use in hormone sampling of individuals and in small fish species where plasma sampling is often terminal and repeated sampling is impossible. In addition, this technique is of particular interest to those who are interested in sampling cortisol from fish. The process of repeated blood sampling (from any size fish) is a stressor and thus cortisol levels obtained from blood often reflect a stressed value instead of a baseline value. Certainly, the transfer from home tank to confinement in a water-born hormone collection vessel represents a potential for an acute stress response [50] However, if it is possible to habituate fish to the water-born hormone collection vessel then it might be possible to obtain both initial acute stress response levels of cortisol as well as repeated measurements of baseline levels of cortisol from a single individual.

Wong et al., (2008) demonstrated that convict cichlids (*Amatitlania nigrofasciata*) could be habituated to this paradigm and show significant correlation between plasma levels and water-born levels of cortisol. A second study attempted to replicate this result in

the sailfin molly (*Poecilia latipinna*) and found a strong correlation between plasma and water born levels but did not see the significant drop in cortisol levels over repeated confinement observed by Wong et al. [41]. To my knowledge these are the only two studies to date that have attempted to validate a habituation paradigm for repeated water-born cortisol sampling in fish.

This technique would be useful in *Xiphophorus birchmanni* as repeated sampling of hormones had previously been impossible due to the small size of this species. The ability to repeatedly sample hormones from this species (a model system in behavioral ecology and ethology) would be of particular utility in answering questions regarding hormonal mechanisms underlying mate-choice. In this study my goal was to examine the feasibility of water-born hormone sampling for *X. birchmanni* as well as to document the range of plasma and water-born cortisol levels for females of this species.

Methods

Animals

Adult female northern swordtails of various ages (*Xiphophorus birchmanni*) were obtained from Calnali, Hidalgo Mexico and housed in the laboratory in mixed-sex groups before the experiment began. Two weeks prior the start of the experiment females were moved into all-female groups of 4 individuals per 10-gallon aquarium maintained at 24C with adequate shelter (hollow brick pavers) and a photoperiod of 12:12 h light:dark. Fish were allowed 2 weeks to acclimate to the new aquaria and feeding schedule. Fish were fed with either bloodworms or frozen brine shrimp once daily after experimental procedures. A total of N=9 adult female *X. birchmanni* were used.

Sample collection

Females were exposed to four successive days of one-hour beaker confinement. Each female was individually transferred from her home tank via hand net to a clean (ethanol and water rinsed) 600 mL beaker with opaque sides (to prevent visual contact between fish) filled with 400 mL of filtered aquarium water (drawn from a reservoir in which no fish had been kept). Females were removed in random order from home tanks and

placed immediately into beakers with a hand net (rinsed with ethanol and water between fish). After transfer females were left undisturbed in a quiet room for one full hour. After one hour, females were removed from the beaker by pouring the water through a net into a second beaker and returned to their home tank. Any irregularities in transfer to or from the home tank were recorded. This procedure was repeated for days 2, 3, and 4. On all four days, the water obtained from individual beakers was immediately filtered via Whatman filter paper 1.

On day 4 water collection was followed by immediate blood collection from all females. Blood was collected via caudal venipuncture with a heparinized 26 gauge syringe, and time to draw blood (time from initial contact with beaker to completion of blood draw) was recorded. Female standard length was also obtained at this time. Once collected, blood was immediately placed on ice until plasma separation. Plasma was separated from blood samples within 1 hour of blood collection.

All hormones were extracted and eluted using a previously established protocol (Wong et al, 2008). Steroid hormones were extracted from diluted (in 4mL distilled water) plasma and from undiluted hormone collection water (400mL) via sep-pak C18 column solid phase extraction following procedures outlined in Wong et al. 2008. Ethyl acetate (4ml) was used as the solvent for elution of steroids from the column. This technique should collect only free (and not glucuronidated or sulfated) steroids [51]. Ethyl acetate was used for both plasma and water sample extraction to yield samples with comparable hormone composition [48,51]. Eluates were dried under nitrogen and re-suspended in 600 microliters EIA buffer. Hormones extracted from plasma were re-suspended in 100X their original plasma volume in 5% ethanol/95% EIA buffer.

According to the manufacturer's (Cayman Chemical item number 500360) specifications kit sensitivity was 35 pg/ml. The specificity of the assay was 100% for cortisol, 4% for prednisolone and 1.6% for cortexolone. All other steroids tested, including corticosterone, cortisone, and testosterone, were less than 0.5%. Intra- and inter-assay variability were 7.4 and 6.7 %CV respectively (manufacturer's data). Standard curve and all plasma and water samples were run in duplicate and read at 405 nm on a Vmax Kinetic ELISA Microplate Reader.

Parallelism validation

The use of commercial cortisol EIA kits for *X. birchmanni* hormone quantification was validated using pooled waterborne cortisol samples from female *X. birchmanni*. This pool was serially diluted to create 6 diluted samples (from 1:2 through 1:64). The dilution curve was parallel to the standard curve. Comparing slopes: t (overall $df=11$) = 0.1737, $p = 0.8653$, critical $t=2.2$ (Fig. 3.1).

Results

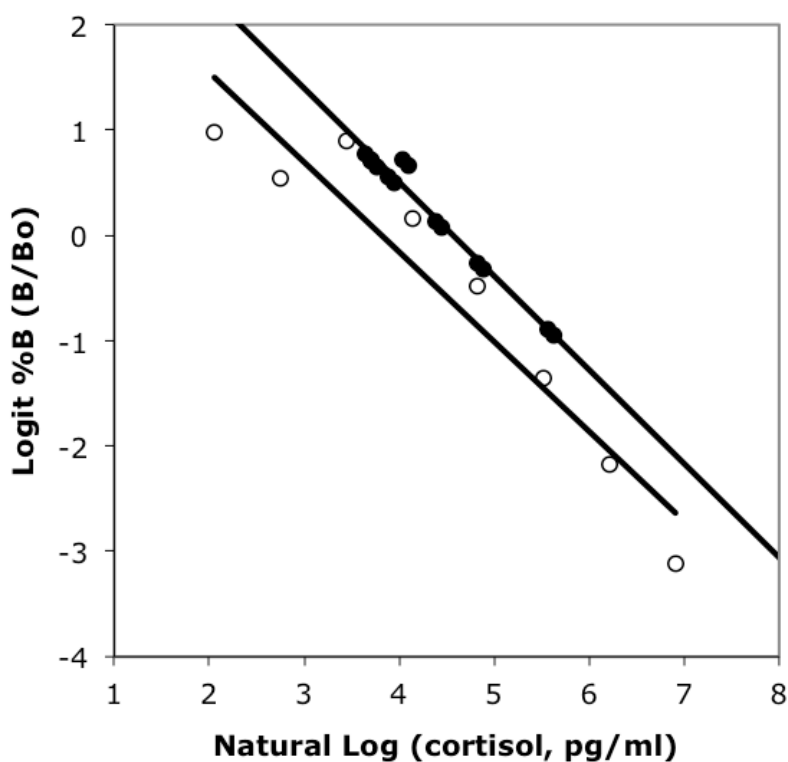


Figure 3.1 Validation of the cayman cortisol EIA kit with *Xiphophorus birchmanni* pooled water samples. Comparing slopes: t (overall $df=11$)=0.1737, $p=0.8653$. Because t -observed is $< t$ -critical (and $p=0.8653$), we accepted the null that the slopes were the same and that parallelism was achieved.

The parallelism assay supported the conclusion that the Cayman Cortisol EIA kit is suitable for the measurement of cortisol in *Xiphophorus birchmanni* [Fig 3.1].

Cortisol release rates over 4 day repeated confinement

Individual *X. birchmanni* water-borne cortisol values ranged from 4901.6 pg/ml to 688.7 pg/ml over four-day repeated testing [Fig 3.2]

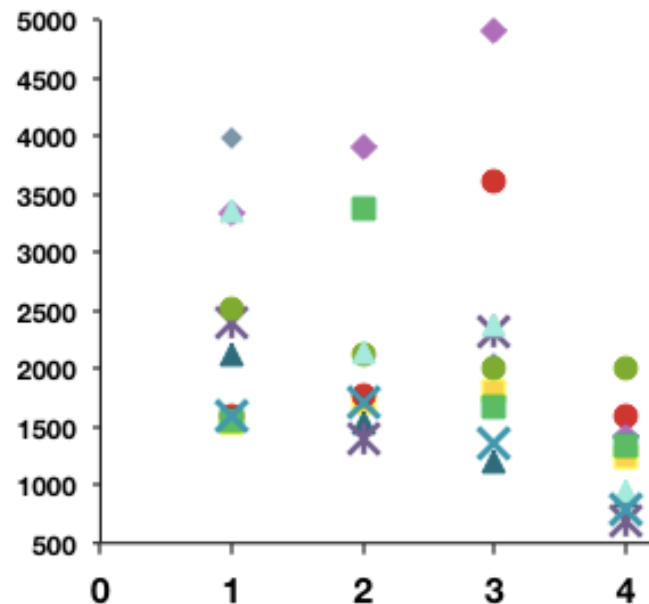


Figure 3.2 Individual water-borne cortisol values over 4 days of repeated confinement.

Mean water cortisol levels decreased from 2398.9 pg/ml on day 1 to 1216.4 pg/ml on day 4, a 49.3% drop in excreted cortisol [Fig 3.3]. There was a significant main effect of day ($F[3,27] = 6.059$, $p = 0.003$) and post-hoc tests revealed that cortisol levels on Day 4 were reduced compared to Day 1, $t(9) = 3.8059$, $p = 0.004$.

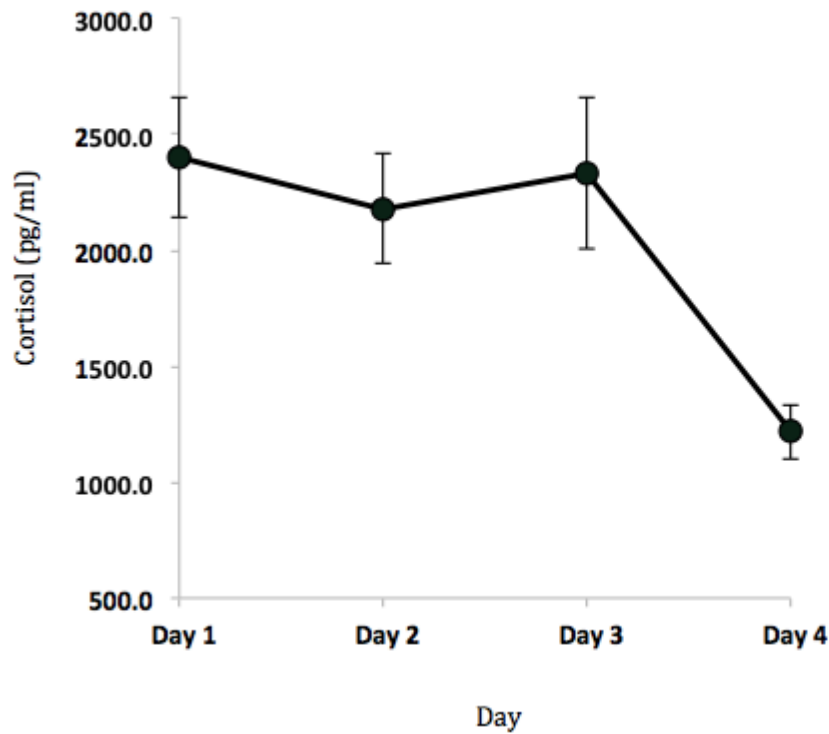


Figure 3.3 Mean water-borne cortisol levels decline over four days of repeated confinement. There was a significant main effect of day, $F(3,27) = 6.059$, $p = 0.003$. * *Post-hoc* tests revealed that cortisol levels on Day 4 are reduced compared to Day 1, $t(9) = 3.8059$, $p = 0.004$

Correlation between water-borne and plasma cortisol levels

The correlation between water and plasma cortisol levels on Day 4 was not significant. This experiment started with N=12 females. I excluded 3 females from this analysis: the first because I did not have standard length data for her (needed to normalize water-borne values) and the other two females because they were statistical outliers. Values for the remaining nine fish ranged from 26436.8 to 52249.5 pg/ml for plasma cortisol levels. Values ranged from 108.74 to 265.15 pg cm⁻¹ h⁻¹ for water-borne cortisol ($R^2=0.1891$ $P=0.2420$) (day 4) [Fig. 3.4].

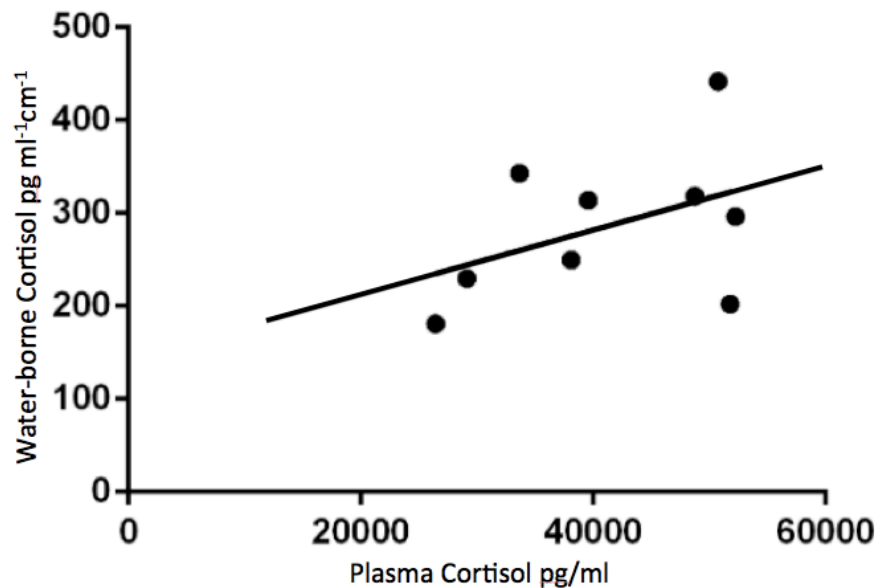


Figure 3.4 No significant relationship detected between water-borne and plasma cortisol levels ($R^2=0.1891$ $P=0.2420$).

Discussion

This study is the first of its kind in *Xiphophorus birchmanni* and an important first step in the characterization of cortisol response for this species as well as in the continued validation of water-born hormone sampling. In this study, I attempted to examine the feasibility of water-born hormone sampling for *X. birchmanni* as well as to document the range of plasma cortisol levels for females of this species. This technique would be of particular utility in *X. birchmanni* as repeated sampling of circulating hormones is difficult because of its size. Blood sampling is therefore most often a terminal procedure.

My experimental protocol was an important first step for validating this technique for use in future studies with this species and closely mirrored those used for other small freshwater species [41,48]. Based on previous studies [48], we expected to observe a decrease in water-born cortisol levels over 4 days and plasma cortisol levels to correlate highly with water-born cortisol levels. In this study, I observed that over the

course of a four-day repeated confinement paradigm female *X. birchmanni* show significant reduction in excreted free cortisol. Most females show a consistent decline in water-borne cortisol levels over 4 days. On Day 3 there were several fish with unusually high cortisol levels. However, plasma levels show a marginally non-significant correlation with water-born levels of cortisol.

The non-significant correlation observed between water and plasma cortisol was particularly surprising because few studies to date have published a non-significant correlation between water and plasma levels of hormones measured. R values observed in other studies with a similar paradigm ranged from $r=0.87$ to $r=0.60$ [41,48]. However, it is important to note that one study found no significant correlation between water-born KT and plasma KT [52].

One possible explanation for the lack of correlation could be sample size. In very small sample sizes, variability in correlation between plasma and water born hormones will have a much greater affect on the goodness of fit to the line of regression (Earley, personal communication). In the present study we used 9 females. Many of the studies that found significant correlations between plasma and water-born steroids had much larger sample sizes. Wong et al., (2008) for example had $n=25$ for their plasma to water-born steroid comparison, Kidd et al., (2010) had $n=23$ and $n=40$ and Ramsey et al. (2011) had $n=21$. If the significance level of the correlation between blood and water-born cortisol were to increase with increased sample size, then this technique would be validated for future use in *X. birchmanni*. If increasing the sample size does not increase the significance level, then these results would also be important by limiting the utility of this technique for researchers using this and closely related species.

A second factor that may have contributed to the low correlation between plasma cortisol and water values is that the individual reproductive states of the females used in this experiment were unknown. Kidd et al., (2010) demonstrated that female reproductive stage can affect correlation between water and plasma values of hormones involved in reproduction. Specifically, they found that in convict cichlids (*A. nigrofasciata*) the correlation between plasma and water values of PGF were significantly lower during the early spawning portion of the reproductive cycle. Given

that cortisol has been shown to play a role in reproduction and has a cyclical pattern of release in live-bearing fishes [37], it is possible that differences in reproductive stage between the females being tested could have influenced the degree of correlation between the plasma and water values.

Without data suggesting a significant correlation between blood and water-born cortisol in this species interpretation of water-born cortisol data has little meaning. However, had the water-plasma cortisol correlation been highly significant (as it might be given an increase in sample size), the range in water-born cortisol release observed in this study is consistent with the range of water values seen from other teleost species [41,48,53] and with the pattern of decline in cortisol levels over repeated confinement seen in other fish species[48], showing a statistically significant decline by day four of the experiment. Day one levels are comparable with day one levels from other small fish in repeated confinement paradigms and to stressed cortisol levels in other teleosts, and day four levels are comparable to low stress levels found in other teleost species [41,48,54].

Interestingly, the drop in cortisol over 4 days observed in this experiment differs from results seen in another poeciliid species (*Poecilia latipinna*) tested in a similar manner [41]. In the *P. latipinna* study, cortisol water levels of repeatedly confined females did not change over four days of confinement. Because cortisol levels did not change, it is not clear whether cortisol excretion was an indicator of high stress or low stress (the authors suggest low stress). Although it is to be expected that even within the same genus fish of different species may display different cortisol profiles [13-18,55], it is interesting to note that the water-born cortisol values reported for *P. latipinna* are consistently higher for all measurements than those observed in our study.

If these levels in *P. latipinna* are actually levels of cortisol secretion associated with a stress response, one potential explanation for the difference between the *X. birchmanni* and *P. latipinna* studies could be the volume of water in the hormone collection beaker. The *P. latipinna* study used 250 ml beakers with 100ml water volume. In setting up this experiment I tested several beaker sizes for the repeated confinement procedure. Initially I used a 200ml beaker with 150 ml of water. In this small volume of water, I found the fish to be significantly more agitated and actively attempting to jump out of the

beaker. When I replaced the 200ml beaker with a 600 ml beaker (400ml water) I found that the fish had far lower levels of apparent agitation and sat quietly for the duration of testing. If the size of the beaker/water volume used in the *P. latipinna* study was sufficiently small to prevent habituation of the stress response then it is possible that replicating the study with larger beaker size would produce a decline in cortisol over repeated confinement.

There are several possible mechanisms that could underlie decreasing cortisol release over repeated confinement: habituation, negative feedback and adrenal exhaustion. In habituation, the females experience an initial stress response to being placed in a novel environment (collection beaker) that gradually subsides over the course of repeated exposure to the environment. Studies across vertebrate taxa have shown that it is possible to habituate the hypothalamic-pituitary-adrenal axis (HPA) or hypothalamic-pituitary-interrenal axis (HPI) over repeated exposure to mildly stressful events/environments. Rees et al., 1985 showed that the corticosterone response in ducks was reduced after repeated exposure to exercise (an inherently stressful stimulus) and exercise plus handling. In the same study, the experimenters injected ducks displaying reduced corticosterone after repeated exposure to exercise with ACTH and observed a rapid response of corticosterone. These data lend support to the hypothesis that the HPA axis was habituated and the decrease in corticosterone response was not due to negative feedback or adrenal exhaustion. Similar results were found in rats [56]. In brown trout, daily exposure to malachite green, an environmental stressor, produced a habituation of the stress response over 4 weeks [57] and in rainbow trout response to daily stress was habituated over 10 weeks [58].

A second possible mechanism underlying the reduction of cortisol over repeated confinement is negative feedback of cortisol on the hypothalamo-pituitary axis. Early studies [59] show that injection of cortisol can produce (presumably via negative feedback onto ACTH production) interrenal atrophy in a dose- and time-dependent manner in the cichlid *Tilapia mossambica*. Cericato et al., 2009 showed that after 96 hours of exposure to agrichemicals, Jundia (*Rhamdia quelen*) display reduced cortisol levels, and when injected with ACTH (ACTH challenge) show significantly reduced cortisol response to ACTH injection as compared to controls. In addition, if a negative

feedback system is the underlying mechanism, one would expect to see increased ACTH in response to decreases in cortisol. Several studies [60] have used interrenal cortisol inhibitors in fish to demonstrate increases in ACTH response.

Finally, adrenal exhaustion, a state well characterized in mammals [61,62] but poorly studied in fish may be occurring. Adrenal exhaustion occurs when an animal is held in a stress-inducing environment and continues to respond by producing cortisol from the adrenals until production of cortisol ceases due to adrenal/interrenal atrophy or unavailability of precursors for hormone synthesis [61]. Given the duration and severity of the applied stressor in this study, adrenal exhaustion seems unlikely.

In order to differentiate between habituation and negative feedback or adrenal exhaustion mechanisms, it would be necessary to show that the drop in cortisol seen in this experiment is carried past the initial day of reduction (Day 4). If, on repeated exposure to the initially stress-inducing environment, cortisol levels remain low, then habituation would be supported. In addition to the maintenance of significantly lower cortisol levels on repeated exposures, it would be necessary to demonstrate that the HPI axis is still reactive via ACTH challenge. If presumably habituated animals injected with ACTH showed increases in cortisol levels, the habituation hypothesis would be supported. If, however, no increase in cortisol occurred in response to ACTH injection, this would further support the adrenal exhaustion hypothesis. If cortisol levels are maintained after extended exposure and if the HPI axis can be activated via ACTH challenge, this provides support for small volume water-borne hormone sampling as a viable technique for repeated measurements of hormones in this species (with the caveat that in experiments seeking baseline or 'unstressed' cortisol levels, fish may need to be habituated to the sampling apparatus prior to collection of experimental data).

CHAPTER IV

CORTISOL AND REPRODUCTIVE STATUS IN A WILD POPULATION

Introduction

Classically, the field of reproductive endocrinology has been centered on understanding the role of sex steroids in reproductive physiology and behavior. However, more recently researchers have begun to recognize the importance of other steroids in reproductive function. Specifically, the corticosteroids have become the focus of many modern studies regarding the regulation of reproduction. Corticosteroid receptors are found in many cell and tissue types throughout the body and thus have the ability to influence myriad physiological processes including metabolic and immune functions. There are two classes of corticosteroids: mineralocorticoids and glucocorticoids. Because of their involvement in modulation of reproductive behavior and physiology across vertebrate taxa I chose to focus on glucocorticoids.

Glucocorticoid receptors have been found in both male and female reproductive tissue across vertebrate taxa and the gonads may actually have the potential to produce corticosteroids themselves, as all necessary enzymes required for corticosteroid synthesis and cortisol as well as 11-deoxycortisol has been detected in both gonads and fluid products of gonads [63]. These results suggest the potential for glucocorticoids to have both inhibitory as well as stimulatory influence on reproductive function. The literature regarding glucocorticoid influence on reproductive function reflects this potential, with many studies reporting conflicting results. The variation in reported roles for glucocorticoids is particularly apparent in the fish literature.

In fish, cortisol is the major glucocorticoid detected in plasma. Cortisol in fish is produced in the interrenal tissue, an adrenal homologue. In contrast to the encapsulated adrenal gland in mammals, fish interrenal tissue is distributed diffusely inside of the anterior portion of the kidney with no distinction between cortical and medullary tissues. However, the major corticosteroids cortisol, cortisone, corticosterone and 11-deoxycortisol seem to be conserved across vertebrate taxa. Increases in plasma cortisol are often associated with external environmental stressors and these stressors have been correlated with deleterious effects on reproductive function. Many

of the deleterious effects associated with stress and with high levels of cortisol are as a result of disruption of vitellogenesis (process of yolk formation in eggs). However, increases in cortisol from basal levels have also been associated with normal reproductive function, for example, in the brown trout, a significant increase in cortisol coincides with ovulation [36,57]. In addition, interactions between sex-steroids and cortisol have been demonstrated. In salmon, gonadectomy actually suppresses interrenal function whereas estrogens and androgens stimulate interrenal activity[64-66]. Notwithstanding an apparent role in oocyte maturation and ovulation, cortisol alone seems to be unable to stimulate these processes, but instead might have a role in sensitizing the ovary to other steroids or gonadotropins [67,68].

Despite the fact that there has been fairly extensive research into the relationships between cortisol and reproductive physiology in fish to date, a major proportion of these studies in fish have been carried out in just a handful of species and a large majority of those are from the genus *Oncorhynchus*, a group of oviparous fish that display external fertilization. Surprisingly, ovoviviparous fish have been the subject of far fewer studies in this area.

In the ovoviviparous guppy *Poecillia reticulata*, cortisol levels are high at the peak of oocyte maturation, drop during the fertilization stage and then rise over the course of gestation until near the time of parturition at which time they display a rapid decline suggesting a role in the maintenance of pregnancy [37,42]. Further supporting this hypothesis, treatment of pregnant guppies with exogenous cortisol resulted in a significant delay in parturition [42]. Ovoviviparous fish present a unique opportunity among fish to investigate the role of glucocorticoids in reproductive physiology and behavior over a longer time scale as these fish are internal fertilizers, have the ability to store sperm for up to 8 months and embryonic development occurs within the female of the species.

As a first step towards understanding the role of cortisol in modulating reproductive physiology and behavior in the ovoviviparous teleost *X. birchmanni*, I asked whether there was a correlation between cortisol and stage of oocyte/embryo maturation in a wild population. I hypothesized that females would display a significant drop in cortisol

levels around the time of fertilization (stage 3) elevated levels of cortisol throughout pregnancy (stages 4-7) with a significant drop in cortisol levels around the time of parturition (stages 8-9).

Methods

Sample collection

I analyzed a total of 19 females from 2 different populations Garces (GARC) (20.5722N, 98.1648W) (n=5) and Coacuilco (COAC) (21.098N, 98.586W) (n=14). Females were collected using minnow traps. A timer was started at the first contact with the trap line. Once the trap was initially contacted and drawn to shore, females were sorted and blood drawn (via rapid decapitation and blood drawn from caudal vein with a heparinized capillary tube) within two minutes (if two minutes was exceeded before the blood could be collected these females were excluded from analysis). Blood was expelled from the capillary tube with a clean infant ear bulb into micro-centrifuge tubes and placed immediately on ice for transport. Standard length data was recorded just before rapid decapitation, total mass (head and body) immediately following, and bodies were fixed in 10% Formalin and then transferred to 75% ethanol for storage at room temperature. Plasma was separated from blood via centrifugation and stored at -20 until analysis. Tissues stored in 75% ethanol were dissected and ovaries removed. Any eggs/embryos present were counted and staged under a dissecting scope according to a 9-stage scale first outlined in Lambert (1970) and Reznick (1980) with modification by Venkatesh et al., (1990)[69].

Hormone analysis

The use of commercial cortisol EIA (Cayman chemicals item number 500360) kits for *X. birchmanni* hormone quantification was validated using pooled hormone from female *X. birchmanni*. This pool was serially diluted to create 6 diluted samples (from 1:2 through 1:64). The dilution curve was parallel to the standard curve (Comparing Slopes: t (overall $df=11$) = 0.1737, $p = 0.8653$, critical $t=2.2$) (fig. 1). See chapter III for kit sensitivity data and parallelism validation. Standard curve and all cortisol samples were

run in duplicate using the protocol described in Chapter III and read at 405 nm on a Vmax Kinetic ELISA Microplate Reader.

Results

Oocytes and embryos were not found at all stages of development. We found oocytes and embryos at stages 2,3,5,6,7 and 9. Brood size ranged from 0 to 36 oocytes/embryos per female. There was a significant correlation between female mass and embryo number ($R^2=6.551$, $P<0.0001$) [Fig4.1] but no significant relationship between plasma cortisol values and embryo number ($R^2=0.06273$, $P= 0.3161$) [Fig. 4.2].

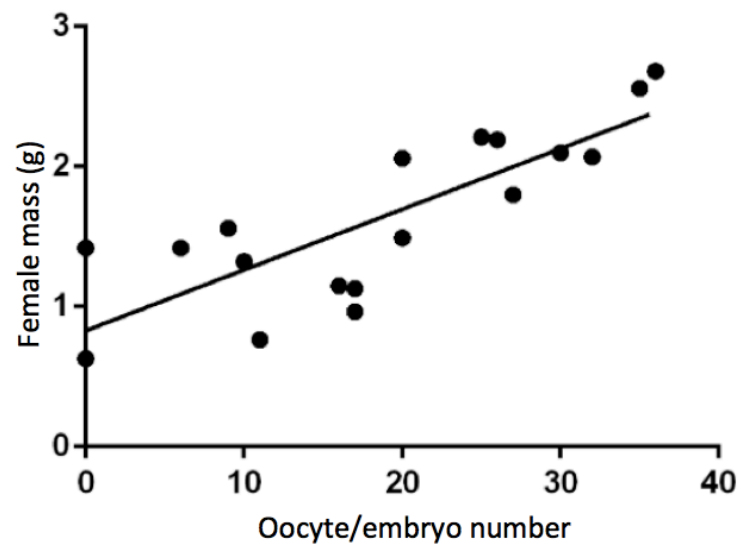


Figure 4.1 Correlation between female mass and embryo number ($R^2=6.551$, $P<0.0001$).

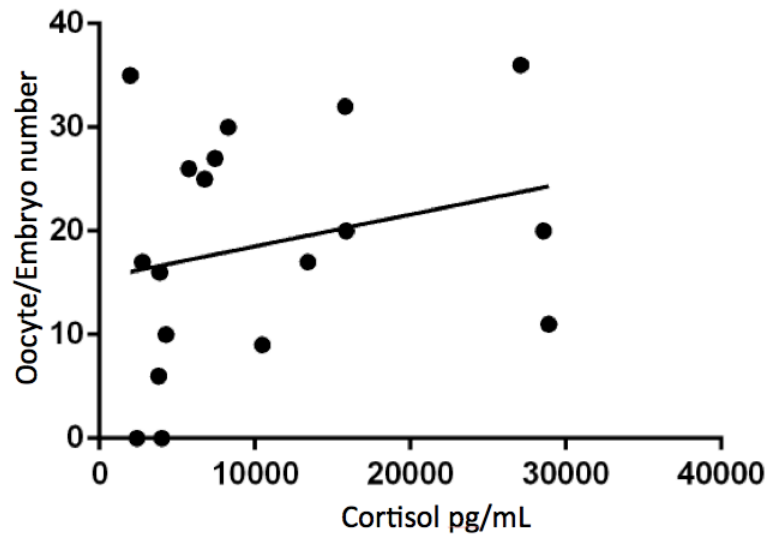


Figure 4.2 Linear regression of plasma cortisol values against oocyte/embryo number revealed no significant relationship ($R^2=0.06273$, $P= 0.3161$).

The total range of cortisol levels for females from both populations was 1980-28900 pg/mL plasma. Overall, there was no significant difference between cortisol levels for females from COAC population and females from GARC population (unpaired t-test: $t(16)=2.544$, $P=0.8024$) The range for COAC was from 1980 to 28570 and for GARC from 2400 to 28900. The relationship between ovarian stage and circulating cortisol levels showed a marked decrease in cortisol values at stages 3 and 9 compared to other ovarian stages. My data showed significantly lower values for stages three (t-test p-value 0.019) and nine (t-test p-value 0.028) compared to stage two.

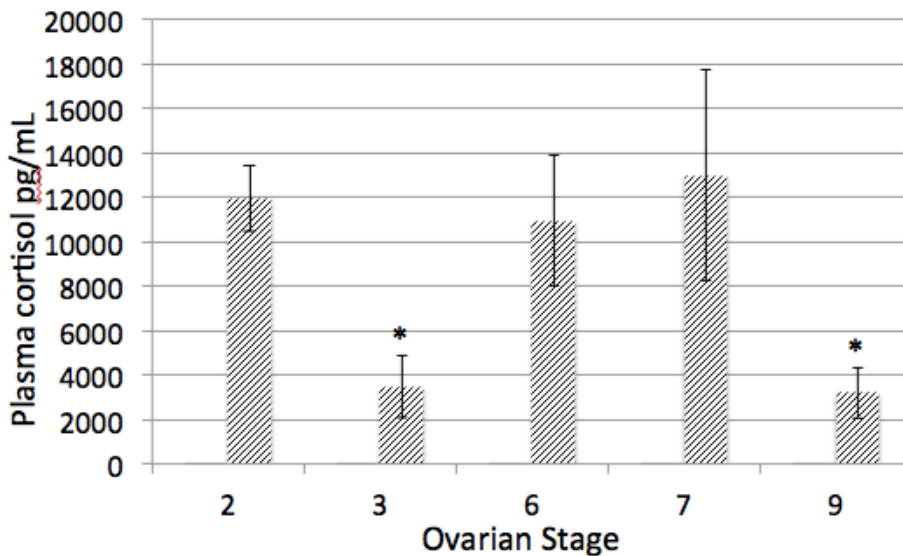


Figure 4.3 Plasma Cortisol levels over the course of the ovarian cycle in *X. birchmanni* show higher levels before and after fertilization with a significant decline around the time of parturition.

Discussion

With this study, I sought to investigate the relationship between plasma cortisol levels and reproductive state in two wild populations of female *X. birchmanni*. This is the first study of its kind in *X. birchmanni*. Based on results from previous studies on fish in the same sub-family [37,42,70] I hypothesized that females would display a significant drop in cortisol levels around the time of fertilization (stage 3) elevated levels of cortisol throughout pregnancy (stages 4-8) with a significant drop in cortisol levels around the time of parturition (stage 9). If cortisol levels are actually indicative of reproductive status and if female reproductive behaviors are correlated with reproductive status in this species, then it is also possible that release of cortisol over the gills could be used as a signal to males that a female is potentially receptive to mating. The lack of a relationship between cortisol and embryo number agrees with previous studies in this genus[37,42].

Cortisol values fell within the range of cortisol levels reported for fish in the family *Poeciliidae* [37]. *P. reticulata* values ranged from 5,000 to 45,000 pg/ml at low stress to

122,000-465,000 pg/ml stressed. The fact that the data from *X. birchmanni* display cortisol values in the very low range of those found for *P. reticulata* could be a species difference, but it could also be related to the cortisol sampling method. In Venkatesh et al. (1990) blood was collected with a capillary tube from the caudal artery after severing the caudal peduncle. This means that it was still possible for the fish to mount a stress response to this method of collection and depending on the duration over which blood collection occurred, the stress response could be detectable in the plasma cortisol sample. Our method, which involved rapid decapitation of the females in less than two minutes, significantly reduced the possibility of detecting the stress response to the blood collection procedure itself.

Even in the absence of some oocyte/embryo stages for *X. birchmanni*, the overall pattern of cortisol levels over the course of the ovarian cycle is strikingly similar to the published data on *P. reticulata*. This lends support to the hypothesis that cortisol may be a key player in oocyte maturation. This hypothesis has been tested in both live-bearing and oviparous fish [43]. The hypothesis regarding pregnancy maintenance is obviously more specific to livebearers among fish. It is possible that cortisol plays a role in live bearing fish that is similar to the role of progesterone in many mammals.

The pattern of release of cortisol also resembles the pattern of release of progesterone in rats and mice. In rats and mice (and many other mammals) progesterone is a pregnancy maintenance hormone that rises steadily over the course of gestation with a sharp drop directly preceding parturition. Also in rats, an increase in progesterone around the time of ovulation stimulates behavioral sexual receptivity and then after copulation and fertilization the prolonged release of progesterone not only maintains pregnancy but inhibits behavioral sexual receptivity [71]. Interestingly, one study in rats found that progesterone receptors were transiently expressed on large follicles within the ovary and that this transient expression was tightly coupled to the surge of luteinizing hormone which triggers ovulation in females [72]. Obviously because progesterone is elevated during periods of both enhanced as well as suppressed sexual receptivity, there must be other factors or fine scale modulation of hormones involved.

I believe that the similarity between these two patterns of hormone release concurrent with developmental stages of oocytes/embryos warrants further investigation. It would be interesting to replicate the *P. reticulata* experiment in which females were injected with cortisol thus delaying parturition. I also think that characterizing female receptivity behaviors during the phases of oocyte development is an important step in bolstering *X. birchmanni* as a model for mechanisms of reproductive behavior as well as population genetics and the evolution of mate choice.

CHAPTER V

SUMMARY

One of the most obvious factors we think of when we think of reproductive behavior and mate choice is the attractiveness of our potential partner. Understanding what is attractive to members of the opposite sex is an important and easily measurable component of understanding sexual selection. Swordtails (Teleostei: *Xiphophorus*) are a long-studied model in the evolution and genetics of sexual communication [11,12]. For this reason many previous studies in this species have focused on using mate choice trials to document population level female preferences and preference functions for male secondary sex traits.

Less often, we think about why, during a given mate choice trial, a female might not choose the universally attractive male, or why she might not make a choice at all. There are many reasons for this, including environmental stress, predation risk, social interactions, male resources and female physiological state. These factors create individual differences in reproductive behavior. As reproductive behavior is a driving force in sexual selection, these individual differences have the potential to influence the strength and direction of sexual selection, which can lead to extreme polymorphism within a species, hybridization and speciation.

To date, in many mate choice studies, the low or non-responding individuals are treated as outliers or noise surrounding the mean and eliminated from analysis [17]. These non-responding females often make up around 25% of the subjects in any given choice trial. However, in order to understand sexual selection we need to understand what traits females prefer, why they prefer those traits, how much variation there is in the preferences (both within and between females) as well as what motivates a female to mate. For this reason, understanding the underlying basis of the behavior of non-responding females will contribute significantly to our understanding of the mechanisms of sexual selection.

I chose to focus on attempting to understand what motivates a female to mate: why do some females choose not to respond and how does this behavior change over time? Because of previous research across taxa revealing relationships between

glucocorticoids and reproductive behavior in other fish species, cortisol emerged as a candidate hormone for synchronizing reproductive physiology with behavior in *X. birchmanni* [37,42]. My study had three objectives: To evaluate possible relationships between cortisol and consistency/repeatability of reproductive behavior in captive animals; to establish the physiological range of cortisol in female swordtails, and to examine the relationship between cortisol levels, and reproductive status in a natural population.

The first study attempted to evaluate possible relationships between cortisol and repeatability and consistency in female mate choice behavior in captive animals. To my knowledge, this was the first study to attempt to assess repeatability and consistency in mate choice among female *X. birchmanni*. Repeatability and consistency of mate choice were both very low and relationships with cortisol were not determined because of problems with the cortisol collection methods. These same factors have been assessed in fish within the same genus, *X. nigrensis* [33]. In this study females were allowed total, barrier-free access or purely visual access to live males. This allowed females to interact with and receive feedback from the males. A primary concern with the animated stimulus mate choice protocol used with the *X. birchmanni* females is that the stimulus males swim on screen, raise their dorsal fins and swim off screen repeatedly. No interaction or feedback is given to the female. It is my understanding that the Rosenthal lab is working toward resolving this issue by developing a system where the stimulus males might be able to change behavior based on feedback from the female. If this system were put into place I think that this would strengthen the validity of the current mate choice trial set up and might make it easier to obtain consistent and repeatable results.

Before moving forward with further experiments regarding repeatability and consistency of mate choice in this species I think it is important to characterize the full repertoire of behaviors involved in courtship. In other species within the genus-like *X. nigrensis*, female behaviors like glides that indicate receptivity toward a male have been documented and extensively characterized both in the wild as well as in the lab. Additionally in *X. nigrensis* it has been validated that association time predicts probability of actual mating[73]. This type of information is undocumented for *X.*

birchmanni. These types of foundational experiments that highlight the time at which females are actually selecting the male that will fertilize their oocytes are critical steps before conducting more complex studies regarding female receptivity and mate choice in this species.

In fact, the main courtship behavior that has been documented in *X. birchmanni* is male-male competition and often times coercive mating in which a male chases a female around until he can get close enough to insert his gonopodium [74]. *Xiphophorus birchmanni* appear to have no observed female receptivity behaviors beyond association time in artificial settings (G. Rosenthal, personal communication). This is not uncommon among poeciliids [74]. Additionally, given the fact that females in this genus can store sperm for up to 8 months, it is possible that post-copulatory sexual selection could be more important in this species than pre-copulatory choice. Post-copulatory choice might also make sense with regards to the ambiguous pattern of female choice seen in this and other species over repeated mate choice testing [75].

One aspect of the generally accepted protocol for mate choice trials that I find particularly interesting is the isolation of females from males prior to testing. Several studies have shown that isolating females from males increases their motivation to mate [74]. However, increased motivation to mate can also be associated with reduction in choosiness. This is one potential explanation for the low consistency seen in my experiment. Females were isolated from males for two weeks prior to testing and then during repeated testing showed no consistent preference, with many alternating between strong preferences for one of the two male phenotypes between days. If the experimenter is truly interested in which male phenotype a female prefers and would mate with in the wild, I think that in the future a more ecologically relevant approach to the mate choice experimental protocol would include housing females with males prior to testing (even if males and females were separated by a barrier).

The goal of the second study was to establish a range of cortisol for *X. birchmanni* and also to validate whether this type of assay is appropriate for this species.

I successfully documented a range of water-borne cortisol levels that fell within the range for other species of fish in the same size class and family. Additionally, it seems

as though the fish acclimate well to the sampling procedure as evidenced by the significant decrease in water-borne cortisol levels from day one to day four of testing. However, the correlation between plasma cortisol and water-borne cortisol was not significant. This was the first time that this protocol had been applied to this species and I believe that given the variability observed, increasing the sample size is necessary to determine whether a significant correlation exists between plasma and water-borne cortisol values.

The goal of the third experiment was to examine the relationship between cortisol levels and reproductive status in a natural population of *X. birchmanni* females. The results indicate that plasma cortisol varies with ovarian stage (stage of maturation of oocytes/embryos) and that these values follow a similar pattern to progesterone in some mammals [37,42,71]. This pattern of release over the course of ovarian stages suggests a potential role for cortisol in oocyte maturation as well as in pregnancy maintenance in *X. birchmanni*. Although preliminary, the results of these experiments help to lay the groundwork for future research regarding the endocrine mechanisms of female reproductive behavior in *X. birchmanni*.

As a whole, these preliminary results along with my suggested protocol modifications provide a foundation that will be necessary for *X. birchmanni* to be developed as a model system for endocrine mechanisms of reproductive behavior. It is clear from the results of Chapter II that female mate choice behavior is variable both between and within individuals of this species and that our understanding of the mechanisms that underlie this variation is crucial to understanding sexual selection in this species. However, protocols for mate choice experiments must be refined in order to incorporate female reproductive status into testing protocols and to more accurately reflect ecologically relevant environmental conditions. Chapter III suggests the potential for repeated water sampling of hormones from *X. birchmanni*. If the plasma to water hormone correlation can be established in this species it would therefore open many doors for understanding how female behavior is related to changes in hormone levels as the results of Chapter IV would for many questions about the role of cortisol in reproductive physiology and behavior in this species.

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